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Poultry-Associated *Salmonella enterica* subsp. *enterica* Serovar 4,12:d:− Reveals High Clonality and a Distinct Pathogenicity Gene Repertoire▽†

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A European baseline survey during the years 2005 and 2006 has revealed that the monophasic *Salmonella enterica* subsp. *enterica* serovar 4,12:d:− was, with a prevalence of 23.6%, the most frequently isolated serovar in German broiler flocks. In Denmark and the United Kingdom, its serovar prevalences were 15.15% and 2.8%, respectively. Although poultry is a major source of human salmonellosis, serovar 4,12:d:− is rarely isolated in humans (approximately 0.09% per year). Molecular typing studies using pulsed-field gel electrophoresis and DNA microarray analysis show that the serovar is highly clonal and lacks genes with known contributions to pathogenicity. In contrast to other poultry-associated serovars, all strains were susceptible to 17 antimicrobial agents tested and did not encode any resistance determinant. Furthermore, serovar 4,12:d:− lacked the genes involved in galactonate metabolism and in the glycolysis and glyconeogenesis important for energy production in the cells. The conclusion of the study is that serovar 4,12:d:− seems to be primarily adapted to broilers and therefore causes only rare infections in humans.

Salmonella spp. are major zoonotic food-borne pathogens which cause outbreaks and sporadic cases of gastroenteritis in humans worldwide (12). Depending on the serovar, cases of salmonellosis can differ substantially in severity (13). The primary sources of salmonellosis are food-producing animals, such as poultry, pigs, and cattle (30). The pathogen is spread by trade in animals and nonheated animal food products (10).

A European baseline survey on the prevalence of *Salmonella* in commercial broiler flocks of *Gallus gallus* in 2005 and 2006 showed that in the European Union (EU), 23.7% of the broiler flocks were *Salmonella* positive (8). However, the *Salmonella* prevalences and serovar distribution varied widely among the EU member states. The five most frequently isolated *Salmonella enterica* serovars in Europe were those classically observed, like serovar Enteritidis (33.8%), serovar Infantis (22.0%), serovar Mbandaka (8.1%), serovar Hadar (3.7%), and serovar Typhimurium (3.0%). In Germany, the flock prevalence of *Salmonella* was 15.0% among the 377 broiler flocks investigated. In contrast to the well-known serovars described above, the predominating serovar was monophasic serovar 4,12:d:−, with a prevalence of 23.6%. This serovar was also isolated in Denmark and the United Kingdom, with prevalences of 15.2% and 2.8%, respectively.

The German Salmonella National Reference Laboratory (NRL-Salmonella) has received 818 isolates of this serovar between 1998 and 2007, with peaks in 2001 (240 isolates) and 2004 (160 isolates), for diagnosis. Since 2005, the number has doubled annually, and the serovar obviously established itself well in poultry production lines. These isolates were found mostly in broilers (78%), occasionally in turkeys (11.6%) and feedstuff (8.4%), and rarely in pigs (1.3%) and cattle (0.6%). In contrast, infections in humans are only sporadic. During the last 10 years (1998 to 2007), the National Reference Centre for Salmonellae and Other Enterics located at the Robert-Koch Institute, Wernigerode branch, has received 55 strains of this serovar from sporadic human cases of salmonellosis and carriers in Germany (W. Rabsch, personal communication). Similarly, in Denmark, only two isolates in 1993 and in 2002 were isolated from humans (E. Møller Nielsen, Statens Serum Institut, Copenhagen, Denmark, personal communication).

Subtyping food-borne pathogens is an approach often applied to facilitate the epidemiological investigation of outbreaks of gastrointestinal disease and to identify the source of entry into the food chain. Several molecular-based tools have been developed to type bacteria genotypically. Pulsed-field gel electrophoresis (PFGE) is currently the method of choice for the molecular subtyping of *Salmonella* serovars. It has been proven to be a useful discriminatory method which was standardized by the PulseNet Consortium (9). However, although this approach is certainly valuable, it does not reveal data on the gene repertoire and biological properties of a strain. To overcome this weakness, whole-genome DNA microarrays have successfully been applied in comparative genomic hybridizations for *Salmonella* (7, 24, 25). However, whole-genome arrays reflect only one genome of one strain. Because of many serovar or strain genome variations described for *Salmonella*, thematic arrays were developed, such as arrays specially targeting genes involved in resistance profiles (2, 17, 32), phage types (23), or serovars (33, 35). A condensed selection of 109 various *Salmonella* genetic markers comprising the detection of flagellar and somatic antigen-encoding genes, important virulence genes, phage-associated genes, and antibiotic resistance determinants have been used to show the usefulness of

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DNA microarrays for the discriminative characterization of *Salmonella* serovars (18).

In this study, we elucidate the contradicting situation between the high prevalence in broilers and source attribution of broiler meat for humans and the low infection rates in humans of the serovar 4,12:d:− by genotypic characterization using PFGE and DNA microarray to determine the clonality, the pathogenic gene repertoire, and resistance determinants. These data give basic information to discuss the hazard potential of this serovar for humans. For that purpose, a new prototype of a *Salmonella* DNA microarray comprising 281 60-mer oligonucleotide probes was developed and validated in house.

MATERIALS AND METHODS

Bacterial strains. Most reference strains used for the validation of the DNA microarray were previously published (18). All strains and their characteristics are listed in Table S1 in the supplemental material. For the genotypic characterization of serovar 4,12:d:−, strains were selected from the collection of the NRL-Salmonella, which were isolated from feed (7 strains), turkeys (5 strains), broilers (24 strains), and pigs (3 strains). In addition, 17 strains isolated from infected humans who suffered from salmonellosis were selected from the collection of the National Reference Centre for Salmonellae and Other Enterics (Robert-Koch Institute, branch Wernigerode, Germany) (Table 1). For the determination of the genotypic relationship of serovar 4,12:d:− to potentially related diphasic serovars, one *S. enterica* serovar Schwarzengrund, two *S. enterica* serovar Duisburg, and two *S. enterica* serovar Stanley strains were selected. These serovars have the same somatic (O) antigen and phase 1 flagellar (H1) antigen but express in addition phase 2 (H2) flagellar antigens (Table 1). Since the H2 flagellar antigen of *S. enterica* serovar Derby can be present or absent, it was included in the study. Additionally, *S. enterica* serovar Livingstone, serovar Infantis, and *S. enterica* serovar Paratyphi B D-tartrate positive (dT+) were selected to analyze their genetic relatedness to serovar 4,12:d:− because they are frequently isolated from broilers in Germany. In humans, the highly prevalent serovars Typhimurium and Enteritidis were added for the analyses of virulence gene determinants.

Serotyping and antimicrobial susceptibility testing. All *Salmonella* strains were serotyped according to the White-Kauffmann-Le Minor scheme (11) by agglutination with O and H antigen-specific sera (Sifin Diagnostics, Berlin, Germany).

Antimicrobial susceptibilities were tested with 17 antimicrobials or antimicrobial combinations, namely, ampicillin, amoxicillin-clavulanic acid, chloramphenicol, ciprofloxacin, colistin, florfenicol, gentamicin, kanamycin, nalidixic acid, neomycin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline, trimethoprim, and sulfamethoxazole/trimethoprim. The tests were performed by determining the MIC using the NCCLS broth microdilution method (22). Breakpoints were adapted from the Clinical and Laboratory Standard Institute (www.clsi.org), the European Committee of Antimicrobial Susceptibility Testing (www.eucast.org), the Antibiotic Resistance in Bacteria of Animal Origin II project (http://cordis.europa.eu/data/PROJ_FP5/ACTIONeqDndSESSIONeq112482005919ndDOCeq169ndTBLeqEN_PROJ.htm), and the Danish Integrated Antimicrobial Resistance Monitoring and Research Programme 2001 (http://www.danmap.org/).

DNA microarray oligonucleotides. A total of 281 oligonucleotide probes were designed using the program Array Designer 4.1 (Premier Biosoft, Palo Alto, CA). Relevant open reading frame sequences were selected from GenBank 166.0 (http://www.ncbi.nlm.nih.gov/GenBank/index.html) and imported into Array Designer 4.1, and a cross homology analysis against the genome sequence of strain *S. enterica* subsp. *enterica* serovar Typhimurium strain LT2 (accession no. NC_003197) was performed. Based on the avoidance of cross homologies, 57- to 60-mer oligonucleotides were designed using the recommended default options for 60-mer oligonucleotides with 73°C ± 5°C melting temperatures; a maximum ΔG for hairpin, −6.0 kcal/mol; a maximum ΔG for self-dimer, −8.0 kcal/mol; and a maximal length of runs/repeats, 5 nucleotides. For five probes, a shorter oligonucleotide length was selected (40 to 45 mer) because the default options were not successful in finding probes. All other settings were not changed. The probes were assigned to seven different marker groups depending on the functionality of the corresponding gene sequence (number of probes): pathogenicity (83), resistance (49), serotyping (33), fimbriae (21), DNA mobility (57), metabolism (21), and prophages (13). Detailed information for each probe can be downloaded from the supplemental material (see Table S2).

In addition, three 60-mer oligonucleotides derived from the *Arabidopsis thaliana* genes RCA (M86720), RCP1 (NM_12175), and PRKASE (X58149) were designed as negative control probes on the microarray.

The oligonucleotide probes were synthesized on a 40-nmol scale with a C6-aminolink modification (Metabion AG, Munich, Germany).

DNA microarray production. The C6-aminolink oligonucleotides were printed on CodeLink-activated slides (GE Healthcare, Munich, Germany) at a concentration of 30 μmol using a QArray mini arrayer (Genetix, New Milton, United Kingdom). A total of 100 mM sodium phosphate (pH 8.5) was used as printing buffer. Two array fields were printed per slide. Each array consisted of two subarrays representing a duplicated set of probes. One subarray contained eight blocks (six columns and eight rows). The last row of each block consistently contained probes representing positive controls (targeting the *ttrC* gene), negative controls (targeting three different *Arabidopsis thaliana* genes), and printing buffer. The diameters of all the spots were approximately 130 μm. The postmicroarray blocking procedure was performed according to the manual instructions provided with the CodeLink-activated slides. The DNA microarrays were stored in a desiccator at room temperature and used within 3 months.

DNA purification. The *Salmonella* strains were grown for 16 to 18 h at 37°C in Luria-Bertani medium with gentle shaking. A 1.6-ml culture aliquot was taken for genomic DNA isolation using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with an elongated lysis time of 3 h. The amounts of DNA were spectrophotometrically determined by measuring the absorption at 230, 260, and 280 nm.

Fluorescence labeling of genomic DNA. The genomic DNA was labeled with Alexa555 or Alexa647 using the genomic labeling kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. Briefly, a 4-μl aliquot containing at least 4 μg DNA was suspended in 20 μl of 5′-Alexa555- or Alexa647-labeled random nonanucleotides in 1× reaction buffer (genomic labeling kit; Invitrogen). The DNA was denatured at 95°C for 10 min and immediately placed on ice. A 5-μl aliquot of a deoxynucleotide mix containing Alexa555-aha-dCTP or Alexa647-aha-dCTP (Invitrogen) and 1.5 μl of the Klenow fragment (40 U/μl) were added. The labeling reaction was incubated at 37°C for 3.5 h. After stopping the reaction by adding a 5-μl aliquot stop buffer, the unincorporated deoxynucleotides and nonanucleotides were removed using the BioPrime purification module kit with PureLink columns (Invitrogen). Finally, the eluted DNA was vacuum dried and stored in the dark on ice until use.

Construction of an internal hybridization control. In order to identify the absence of individual probes on the array field potentially caused by print errors, an internal hybridization control (IHC) was prepared as follows: 2-μl aliquots of each of the 281 oligonucleotide probes and the negative control probes (100 μmol each probe) were pooled, and a 24-μl aliquot of this mixture was labeled with Alexa647 dye using the genomic labeling kit (Invitrogen). The dried elute was resuspended in 300 μl hybridization buffer, and a 0.8-μl aliquot was used for the hybridization.

Hybridization and posthybridization washing. Vacuum-dried Alexa555-labeled genomic DNA was resuspended in 30 μl prewarmed hybridization buffer containing 12 μl formamide, 3 μl 50× Denhardt's solution (Fluka, Basel, Switzerland), 3 μl 10% (wt/vol) sodium dodecyl sulfate, 4.5 μl 20× SSC (3 M NaCl, 0.3 M sodium citrate [pH 7.0]), and 7.5 μl 20% (wt/vol) dextrane sulfate. The sample was denatured at 98°C for 2 min and briefly centrifuged, and a 0.8-μl aliquot of the Alexa647-labeled print control was added. The suspension was carefully added onto the *Salmonella* array, under a 22- by 27-mm coverslip (Erie Scientific, Portsmouth, NH) covering the array field. The hybridization was performed for 18 to 20 h at 42°C using a Slide Booster SB401 (Implen, Munich, Germany). Three-second intervals of sonic waves and 7-s pauses were selected to mix the sample during hybridization. For humidity equilibration per incubation chamber, two 500-μl aliquots of double-distilled water were used. After hybridization was performed, the coverslips were removed by dipping the slides in a Schiefferdecker-type glass chamber filled with 200 ml of 33°C-preheated washing buffer 1 (1× SSC, 0.3% [wt/vol] sodium dodecyl sulfate). Further washing steps were performed using an Adawash AW400 washing station (Implen). The slides were placed in the slide tube holder and washed for 3 min at 33°C in approximately 200 ml washing buffer 1 with agitation by pulsed pumping. After a second wash for 3 min with washing buffer 2 (0.2× SSC), a final washing step was performed using washing buffer 3 (0.05× SSC). The slides were dried by gentle centrifugation for 3 min at 3,000 × *g* and stored until scanning with protection from light in the desiccator at room temperature. Per strain DNA was labeled with Alexa555 and hybridized in the presence of Alexa647-labeled IHC. Furthermore, DNA was labeled with Alexa647 and hybridized in the absence of the IHC. Consequently, four spot intensities were generated for each probe.

TABLE 1. *Salmonella* strains used in this study and their characteristics

BfR[a] no.	Serovar[b]	Phage type	Source	Yr of isolation	Array type[c]	PFGE type[d]	Resistance[e]
94-01172	4,12:d:−		Broiler	1994	1	5	Susceptible
98-02398	4,12:d:−		Broiler	1998	1	8	Susceptible
02-02938	4,12:d:−		Broiler	2002	ND	7	Susceptible
02-04641	4,12:d:−		Broiler	2002	ND	10	Susceptible
03-01904	4,12:d:−		Broiler	2003	ND	6	Susceptible
03-02117	4,12:d:−		Broiler	2003	1	2	Susceptible
03-03421	4,12:d:−		Broiler	2003	ND	1	Susceptible
04-00176	4,12:d:−		Broiler	2004	ND	1	Susceptible
04-00981	4,12:d:−		Broiler	2004	ND	6	Susceptible
04-02236	4,12:d:−		Broiler	2004	ND	3	Susceptible
04-02830	4,12:d:−		Broiler	2004	1	1	Susceptible
05-04891	4,12:d:−		Broiler	2005	1	1	Susceptible
05-05163	4,12:d:−		Broiler	2005	ND	1	Susceptible
06-01203	4,12:d:−		Broiler	2006	1	1	Susceptible
06-01260	4,12:d:−		Broiler	2006	ND	1	Susceptible
06-01480	4,12:d:−		Broiler	2006	ND	1	Susceptible
06-01642	4,12:d:−		Broiler	2006	1	1	Susceptible
06-04044	4,12:d:−		Broiler	2006	ND	1	Susceptible
07-01001	4,12:d:−		Broiler	2007	ND	1	Susceptible
07-02442	4,12:d:−		Broiler	2007	ND	1	Susceptible
07-04661	4,12:d:−		Broiler	2007	ND	3	Susceptible
08-01120	4,12:d:−		Broiler	2008	ND	1a	Susceptible
08-01322	4,12:d:−		Broiler	2008	ND	3	Susceptible
08-01574	4,12:d:−		Broiler	2008	ND	1	Susceptible
02-02394	4,12:d:−		Feed	2002	ND	2	Susceptible
04-00098	4,12:d:−		Feed	2004	2	9	Susceptible
05-04943	4,12:d:−		Feed	2005	1	1	Susceptible
06-01412	4,12:d:−		Feed	2006	2	1	Susceptible
07-00780	4,12:d:−		Feed	2007	ND	6	Susceptible
07-01353	4,12:d:−		Feed	2007	ND	1	Susceptible
07-01533	4,12:d:−		Feed	2007	1	1	Susceptible
08-04702	4,12:d:−		Human	2000	ND	5	Susceptible
08-04696	4,12:d:−		Human	2001	ND	4a	Susceptible
08-04701	4,12:d:−		Human	2001	ND	4	Susceptible
07-02168	4,12:d:−		Human	2002	1	4	Susceptible
07-02169	4,12:d:−		Human	2002	1	2	Susceptible
07-02171	4,12:d:−		Human	2002	1	2	Susceptible
08-04694	4,12:d:−		Human	2002	ND	2	Susceptible
08-04695	4,12:d:−		Human	2002	ND	4	Susceptible
08-04699	4,12:d:−		Human	2002	ND	2a	Susceptible
08-04700	4,12:d:−		Human	2002	ND	2	Susceptible
07-02170	4,12:d:−		Human	2003	1	2	Susceptible
08-04698	4,12:d:−		Human	2003	ND	4	Susceptible
07-02167	4,12:d:−		Human	2004	1	3	Susceptible
07-02166	4,12:d:−		Human	2005	1	2	Susceptible
08-04703	4,12:d:−		Human	2005	ND	1	Susceptible
08-04697	4,12:d:−		Human	2006	ND	1	Susceptible
07-02165	4,12:d:−		Human	2007	1	3	Susceptible
04-01557	4,12:d:−		Pig	2004	1	5	Susceptible
04-02779	4,12:d:−		Pig	2004	ND	5	Susceptible
05-03032	4,12:d:−		Pig	2005	ND	2	Susceptible
98-01070	4,12:d:−		Turkey	1998	1	7	Susceptible
02-00476	4,12:d:−		Turkey	2002	ND	4	Susceptible
03-01731	4,12:d:−		Turkey	2003	ND	6	Susceptible
07-02602	4,12:d:−		Turkey	2007	ND	1a	Susceptible
04-00787	4,12:d:−		Turkey	2004	1	3	Susceptible
05-04338	Schwarzengrund		Turkey	2005			Susceptible
04-02395	Stanley		Pig	2004			Susceptible
05-03027	Stanley		Pig	2005			Susceptible
03-03327	Duisburg		Broiler	2003			Susceptible
03-03328	Duisburg		Broiler	2003			Susceptible
07-02558	Derby		Human	2007			Susceptible
07-02554	Derby		Pig	2006			AMP, CHL, STR, SXT, SPE, TET
07-02552	Typhimurium	DT12	Human	2005			Susceptible
07-02553	Typhimurium	DT12	Human	2005			Susceptible
07-02551	Typhimurium	DT120	Human	2006			AMP
07-02560	Typhimurium	DT120	Human	2006			AMP, STR, SMX, TET
07-01996	Typhimurium	DT104L	Broiler	2005			AMP, CHL, FLO, NAL, SMX, STR, SPE, TET
05-05227	Paratyphi B dT+		Broiler	2005			AMP, NAL, STR, SMX, SPE, SXT, TET, TMP
06-02243	Paratyphi B dT+		Broiler	2006			AMP, NAL, SMX, SPE, SXT, TET, TMP
07-01993	Livingstone		Broiler	2004			AMP, STR, SMX, SPE, SXT, TMP
07-01994	Livingstone		Broiler	2006			Susceptible
04-03524	Infantis		Broiler	2004			Susceptible
07-02562	Infantis		Human	2007			Susceptible
07-02000	Enteritidis	PT4	Broiler	2002			Susceptible
07-02001	Enteritidis	PT1	Broiler	1999			Susceptible

[a] BfR, Bundesinstitut für Risikobewertung.

[b] Antigenic formulas are as follows: serovar Schwarzengrund, 4,12:d:1,7; serovar Stanley, 4,12:d:1,2; serovar Duisburg, 4,12:d:enz$_{15}$; serovar Derby, 4,12:f,g:1,2; serovar Typhimurium, 4,12:i:1,2; serovar Paratyphi B dT+, 4,12:b:1,2; serovar Livingstone, 6,7:14:d:l,w; serovar Infantis, 6,7,14:r:1,5; and serovar Enteritidis, 9,12:g,m:−.

[c] Array type only assigned for serovar 4,12:d:−. ND, not determined.

[d] XbaI restriction.

[e] AMP, ampicillin; CHL, chloramphenicol; FLO, florfenicol; KAN, kanamycin; NAL, nalidixic acid; SPE, spectinomycin; STR, streptomycin; SMX, sulfamethoxazole; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TMP, trimethoprim.

TABLE 2. Oligonucleotide probes to detect the *ttrC* gene sequence in serovar Typhimurium

Sequence[a]	% Similarity	Ratio (± SD[d])
ATGACGCATTCACTCATCATTGAAGAAGTGCTGGCTCACCCGCAGGACATTAGCTGG	100	1.00
TACCTTGGAAGGTAGTTCATTGAAGAAGTGCTGGCTCACCCGCAGGACATTAGCTGG	72	0.423 ± 0.06
ATGACGCATTCACTCATCATTGAAGAAGTGCTGGCTCACCC**AGGCTTGCAAGCTACC**	72	0.713 ± 0.04
ATGACGCATTCACTCATCATT**CTTCTTCACGACCGAG**ACCCGCAGGACATTAGCTGG	72	0.853 ± 0.19
AT**C**A**G**GCA**A**T**G**AC**A**C**T**TCATA**G**T**A**GA**T**GTG**G**T**C**GC**A**GAC**G**CGCA**C**GA**G**ATTA**C**CT**C**G[b]	70	0.061 ± 0.02
AT**C**ACG**G**ATTC**T**CTCAT**G**ATTG**T**AGAAG**A**GCTCGC**T**CAC**G**CGCAC**G**ACATTAC**C**TG**C**[b]	81	0.268 ± 0.04
ATGACGCA**A**TCACTCAT**G**ATTGAAGAA**C**TGCTGGCTC**T**CCCGCAGGA**G**ATTAGCTG**C**[b]	89	0.066 ± 0.02
AT**A**AC**T**CA**G**TC**G**CT**A**ATT**A**TC**G**AC**G**AC**G**TA**C**TGGC**G**CAC**A**CG**T**AG**T**AC**G**TT**C**GC**C**GG[c]	70	0.066 ± 0.02
ATGA**T**GCAT**G**CACT**A**ATCA**G**TGAA**T**AAGT**A**CTGG**A**TCAC**T**CGC**C**GGAC**G**TTAGC**C**GG[c]	81	0.257 ± 0.13
CTGACGCA**G**TCACTCAT**A**ATTGAAGAA**T**TGCTGGC**T**CGCCCGCAG**G**ATATTAGCTGG[c]	89	0.575 ± 0.04

[a] Nucleotide changes to the original *ttrC* probe sequence are indicated in bold.
[b] Purine bases in nucleotides were exchanged by pyrimidine bases.
[c] Pyrimidine bases in nucleotides were exchanged by purine bases.
[d] SD, standard deviation.

Data analysis. The hybridized slides were scanned with a GenePix 4000B laser scanner (Axon, Foster City, CA) using a resolution of 10 μm. Usually the photomultiplier tube (PMT) gain of both channels was set to 600. Fluorescent images were captured in multi-image-tagged file formats and analyzed with GenePix Pro 6.1 software (Axon). For the normalization of the Alexa555 probe signals detectable in the Cy3 channel of the scanner, a ratio was calculated as follows: the median spot intensity of each probe with the local background subtracted was divided by the median spot intensity of the positive control probe, the *ttrC* gene, with the local background subtracted, which is present in all *Salmonella enterica* subspecies *enterica* serovars (19). The probe control detectable in the Cy5 channel of the scanner was used to highlight absent (e.g., not printed) probes. Probes lacking an Alexa647 fluorescence signal below 100 units were considered print errors and were excluded from the analysis. Based on the average of the spot intensities of all the negative target probes for serovar Typhimurium strain LT2, probe signals for which the ratio was equal to or greater than 0.25 were considered positive. Ratio values between 0.25 and 0.15 were classified as "uncertain." The assignment of an "uncertain" probe signal to the presence or absence of the target was individually decided, either on the basis of additional PCR results performed to this target or after a repetition of the hybridization experiment. For the *Escherichia coli* reference strain EC227, an artificial value of 10.000 units Alexa555 fluorescence raw intensity for the *ttrC* probe was applied for the normalization because the *ttrC* probe gave no signal. The normalized presence/absence data for each strain were imported into BioNumerics (version 5.1; Applied Maths, Sint-Martens-Latem, Belgium) as character values. A cluster calculation analysis was performed with the simple matching binary coefficient using the unweighted-pair group method with arithmetic averages (UPGMA dendrogram type). The maximum parsimony cluster analysis was performed with 1,000 bootstrap cycles, and the exported rendered tree was performed with hidden branches and distance labels shorter than or equal to 1 and rooted tree type.

Validation of microarray signals by PCR. PCRs were performed for the target genes indicated in the supplemental material (see Table S2). The PCR primers were designed by Array Designer 4.1 (Premier Biosoft, Palo Alto, CA) and resulted in 400- to 500-bp amplification products. For the detection of the most antibiotic resistance genes, published primer sequences from various sources were used. A complete list of PCR primers, PCR product sizes, references, and their characteristics is available in the supplemental material (see Table S2). A typical 25-μl PCR contained 0.4 μM of each primer, 200 μM each of deoxynucleoside triphosphate and 1× PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 1.5 mM $MgCl_2$, and 0.75 U *Taq* polymerase (Invitrogen), and 5 ng of the identical DNA preparation was used for the Alexa555/Alexa647 labeling of each strain. The incubation conditions were 95°C for 1 min, followed by 33 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. A 10-μl aliquot of a PCR product was loaded on a 1.5% agarose gel, and an electrophoresis at 6 V cm^{-1} for 90 min was performed. The presence of a clear fragment with the correct amplification size after staining the gel in ethidium bromide was assessed as a positive signal (presence of the gene).

Evaluation of probe specificity. For the assessment of the probe specificity, nucleotides within the positive control oligonucleotide probe sequence specifically detecting the *ttrC* gene in *Salmonella* spp. were exchanged at various positions and with different similarities (Table 2). Nine different polymorphic patterns were designed, including continuing 16-mer nucleotide exchanges at different positions of the sequence and exchanges distributed over the complete 60-mer sequence with homologies of 70%, 80%, and 90%. Nucleotide changes did not modify the melting temperature of the original probe sequence. The set of polymorphic oligonucleotides was printed on CodeLink-activated slides. Alexa647-labeled serovar Typhimurium DNA (strain 51K61) has been hybridized according to the protocol described above, and a mean ratio value of the spot intensities for each polymorphic probe sequence compared to the 100% complementary *ttrC* probe sequence based on eight independent experiments has been calculated as described above.

PFGE. PFGE using the restriction enzyme XbaI (Roche Diagnostics, Mannheim, Germany) was performed according to the standardized PulseNet *Salmonella* protocol (26). Thiourea (100 μM; Roth, Karlsruhe, Germany) was added to the running buffer (0.5× Tris-borate EDTA buffer) to prevent DNA degradation. Strains were assigned to the same PFGE profile type when they showed visually the same fragment sizes between the marker fragment sizes of 1,135 kb and 33 kb. A letter followed by the profile number indicates an additional fragment within the PFGE pattern compared to the profile number without the letter.

RESULTS

Analysis of serovar 4,12:d:− using PFGE. Thirteen different XbaI PFGE profiles were recognized within the 56 serovar 4,12:d:− strains analyzed (Fig. 1). All profiles showed highly similar patterns. The most prevalent profile was PFGE profile 1 (19 strains) followed by PFGE profile 2 (9 strains) (Table 1). Compared to profile 1, the other profiles differed in one to five fragments. PFGE profile 1 was mainly found in broiler and feed isolates but occurred also in 2 out of the 17 isolates from humans investigated. PFGE profile 2 was found in one isolate from a broiler, in one from feed, in one from a pig, and in six from humans. More rarely, PFGE profile 3 was detected in isolates from broilers, turkeys, and humans. All serovar 4,12:d:− PFGE profiles showed low similarities to serovars Duisburg, Schwarzengrund, and Stanley, harboring the same somatic (O) antigen and phase 1 flagellar (H1) antigens, but expressed in addition phase 2 (H2) flagellar antigen, and serovar Derby expressed a variable H2 flagellar antigen (Fig. 1).

Validation of the DNA microarray. The microarray contained 276 57- to 60-mer and 5 40- to 45-mer oligonucleotide probes detecting the presence or absence of genes associated with the pathogenicity of *Salmonella*, such as type 1 or type 3 secretion systems, outer membrane proteins, secreted proteins, Vi antigen-encoding genes, or virulence markers located in prophages. Furthermore, markers for antibiotic resistance determinants, fimbriae, prophage genes, phase-1 and phase-2 flagellar antigens, lipopolysaccharide (O) antigens, insertion

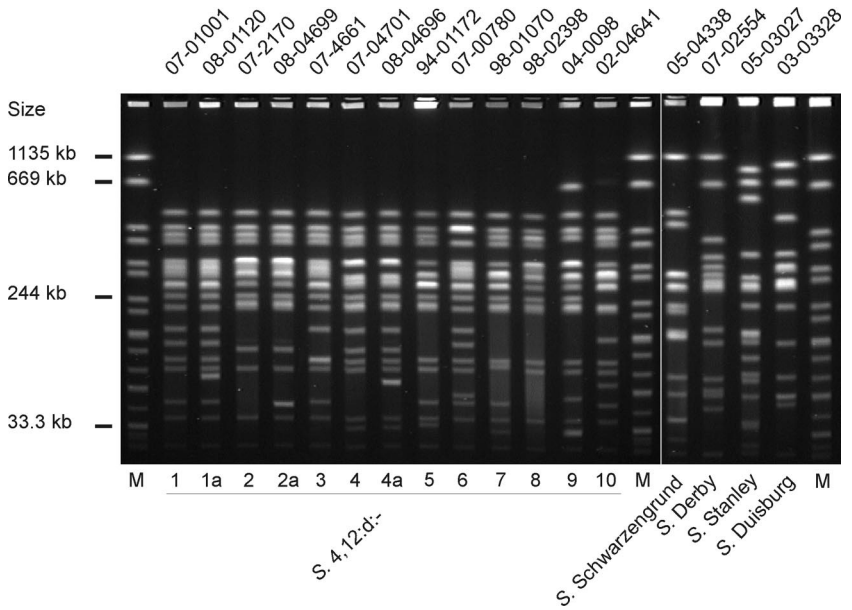
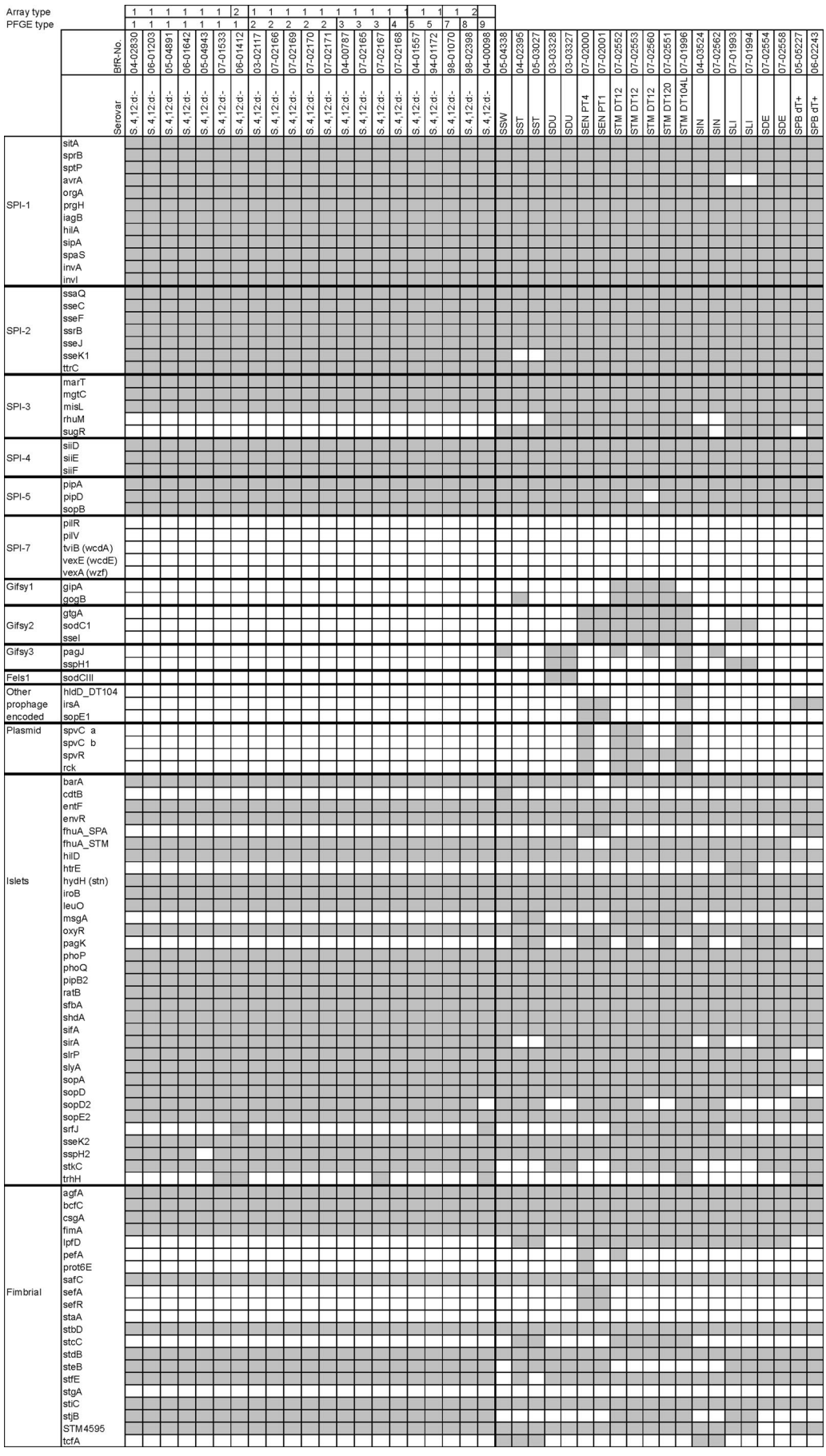


FIG. 1. PFGE profiles of representative *S. enterica* subsp. *enterica* strains after digestion with XbaI. As a molecular weight standard (M), *S. enterica* serovar Braenderup reference strain H9812 was used (lanes 1, 15, and 20). Lanes 2 to 14 represent the 13 different PFGE types of serovar 4,12:d:−. Lanes 16 to 19 represent the PFGE patterns of selected strains/serovars possessing a related antigenic formula to serovar 4,12:d:− (serovar Schwarzengrund, serovar Derby, serovar Stanley, and serovar Duisburg,). Above the gel, the strain numbers are indicated.

sequence (IS) elements, plasmid incompatibility groups, and metabolism were covered by the microarray (see Table S2 in the supplemental material). For validation, 24 reference strains were selected and tested for the presence/absence of the 281 oligonucleotide probe targets by hybridization to the microarray. The data obtained were compared to corresponding PCR amplifications. Altogether, for 256 target genes, the primers were designed or selected from literature for the PCR screening of 23 *Salmonella* reference strains and one *E. coli* reference strain (see Table S1 in the supplemental material). Probes of the serotyping marker group (targeting the *fliC*-, *fljB*-, and *rfb*-specific sequences) were phenotypically confirmed by serotyping according to the White-Kauffmann-Le Minor scheme (11). All negative controls derived from three *A. thaliana* genes and printing buffer showed ratios below 0.1 (data not shown) and were consistently classified negative. The comparison between the microarray and PCR results obtained from the 23 *Salmonella* reference strains gave an agreement of 96.4% (6,227 data signals). The remaining 3.6% (223 data signals) gave inconsistent PCR and microarray results, of which 2.2% (143 data signals) were classified as uncertain (see Table S3 in the supplemental material). The disagreements occurred primarily with probes linked to mobile elements (88 data signals). Often probes gave positive signals, whereas the corresponding PCR products were negative. Apparently, the probes derived from mobile elements might give cross signals with other homologous mobile elements. Possibly, PCR primers did not result in an expected PCR product, because of polymorphisms in the primer binding sequence or because of a truncated gene. Experiments based on various nucleotide exchanges of the control probe *ttrC* showed that the sequence can differ up to approximately 20% with the complementary *Salmonella* target DNA in order to generate a spot intensity after hybridization which is classified as present (ratio ≥ 0.25) (Table 2). All printed oligonucleotide probes have been reliably detected in the Cy5 channel if the IHC has been added to the hybridization reaction. Coincidentally, nonprinted oligonucleotides generated no signals.

Virulence determinant characterization of serovar 4,12:d:−. Most *Salmonella* serovars can express two different antigenic flagella phases. The phase 2 flagellar locus is possibly used in particular environmental circumstances and host-defense mechanisms (20). The microarray results revealed that all serovar 4,12:d:− isolates were monophasic because the phase 2 flagellar-encoding region harboring the regulatory genes *hin* and *fljA* as well as the *fljB* gene encoding the structural filament unit was consistently absent. In addition to investigating flagellar-related genes, the DNA microarray was used to investigate 104 virulence determinants (83 pathogenicity and 21 fimbrial markers) of serovar 4,12:d:−. Among the 21 serovar 4,12:d:− strains, two virulence array types (VATs) were defined (Table 1). A new type has been assigned if the number of virulence determinants analyzed by the microarray differed in more than one marker compared to the predominant virulence determinant pattern recognized in serovar 4,12:d:− strains. VAT 1 was the predominant type with 90% (19 strains) and was found in all sources of isolates from feed, turkeys, broilers, pigs, and humans (Fig. 2). VAT 2 was exclusively found in two feed isolates. VAT 2 differed from VAT 1 in two virulence determinants. In both VAT 2 strains, *trhH* encoding a pilus assembly protein and *srfJ* encoding a glucosyl ceramidase were present, whereas in the second VAT 2 strain, *sopD2* was additionally absent.

The following observations are remarkable in regard to the presence/absence of the virulence determinants. Virulence genes previously described to be located within prophages,


Array type
PFGE type
BfR-No.
Serovar
S. 4,12:d:-
04-02830
06-01203
05-04891
06-01642
05-04943
07-01533
06-01412
03-02117
07-02166
07-02169
07-02170
07-02171
04-00787
07-02165
07-02167
07-02168
04-01557
94-01172
98-01070
98-02398
04-00098
05-04338
04-02395
05-03027
03-03328
03-03327
07-02000
07-02001
07-02552
07-02553
07-02560
07-02551
07-01996
04-03524
07-02562
07-01993
07-01994
07-02554
07-02558
05-05227
06-02243
SSW
SST
SST
SDU
SDU
SEN PT4
SEN PT1
STM DT12
STM DT12
STM DT12
STM DT120
STM DT104L
SIN
SIN
SLI
SLI
SDE
SDE
SPB dT+
SPB dT+
SPI-1
sitA
sprB
sptP
avrA
orgA
prgH
iagB
hilA
sipA
spaS
invA
invI
SPI-2
ssaQ
sseC
sseF
ssrB
sseJ
sseK1
ttrC
SPI-3
marT
mgtC
misL
rhuM
sugR
SPI-4
siiD
siiE
siiF
SPI-5
pipA
pipD
sopB
SPI-7
pilR
pilV
tviB (wcdA)
vexE (wcdE)
vexA (wzf)
Gifsy1
gipA
gogB
Gifsy2
gtgA
sodC1
sseI
Gifsy3
pagJ
sspH1
Fels1
sodCIII
Other prophage encoded
hldD_DT104
irsA
sopE1
Plasmid
spvC a
spvC b
spvR
rck
Islets
barA
cdtB
entF
envR
fhuA_SPA
fhuA_STM
hilD
htrE
hydH (stn)
iroB
leuO
msgA
oxyR
pagK
phoP
phoQ
pipB2
ratB
sfbA
shdA
sifA
sirA
slrP
slyA
sopA
sopD
sopD2
sopE2
srfJ
sseK2
sspH2
stkC
trhH
Fimbrial
agfA
bcfC
csgA
fimA
lpfD
pefA
prot6E
safC
sefA
sefR
staA
stbD
stcC
stdB
steB
stfE
stgA
stiC
stjB
STM4595
tcfA

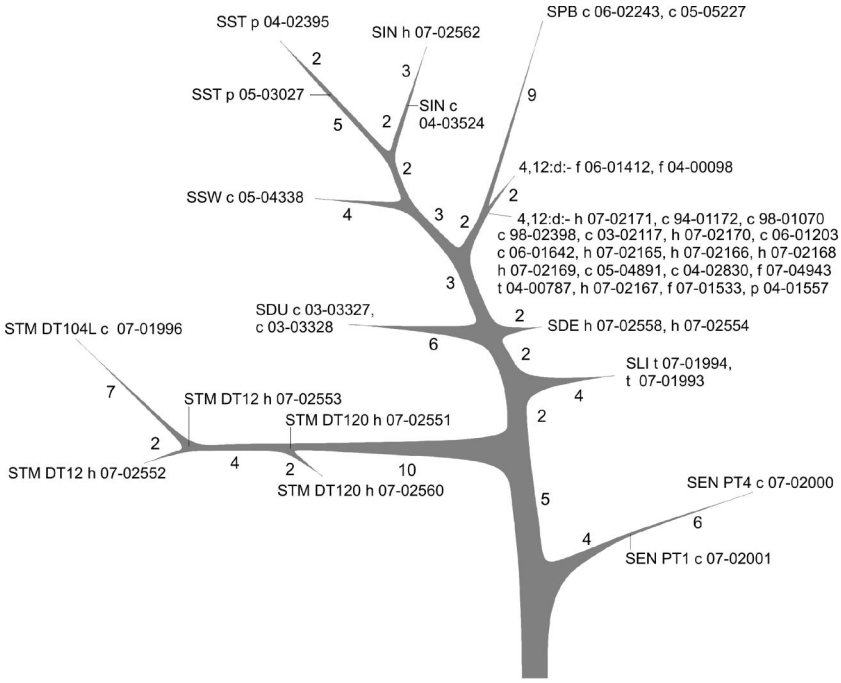


FIG. 3. Rendered maximum parsimony tree. The tree shows the differences between the *Salmonella* isolates based upon 104 virulence determinants. The count indicates the number of genes different between the branches. Strain information is given in the order serovar, isolation origin, and strain number. Abbreviations: SSW, serovar Schwarzengrund; SST, serovar Stanley; SDU, serovar Duisburg; SEN, serovar Enteritidis; STM, serovar Typhimurium; SIN, serovar Infantis; SLI, serovar Livingstone; SDE, serovar Derby; SPB dT+, serovar Paratyphi B dT+; h, human; t, turkey; c, broiler; p, pig; f, feed.

including Gifsy1, Gifsy2, Gifsy3, and Fels1, were absent in all isolates. Within *Salmonella* pathogenicity island 3 (SPI-3), the genes *rhuM* and *sugR* (the 3′ region of SPI-3) were absent in all serovar 4,12:d:− strains. This truncation has formerly been described for other serovars (1). The presence of a virulence plasmid as described for several serovars (5) that could not be recognized was confirmed by the absence of the genes *spvC*, *spvR*, *rck*, *pefA*, and *traT* and the incompatibility marker FIIs. Twelve out of 21 detectable fimbrial markers could be found in serovar 4,12:d:−. The long polar fimbriae (*lpfD*) usually found in serovar Typhimurium and serovar Enteritidis were absent.

Comparison of serovar 4,12:d:− virulence determinants to other serovars. The pathogenicity markers of serovar 4,12:d:− were compared to those of various other serovars expressing the same O antigens commonly associated with poultry. Three of the serovars, Duisburg (4,12:d:e,n,z_{15}), Stanley (4,12:d:1,2), and Schwarzengrund (4,12:d:1,7), share with serovar 4,12:d:− the same O and H1 antigens but express different H2 antigens. Serovar 4,12:d:− and serovar Derby (4,12:f,g:[1,2]) shared the highest number of identical virulence determinants (Fig. 3). Only 7 of the 104 markers determined differed between both serovars. Four genes, *pagK*, *rhuM*, *sugR*, and *lpfD*, were present additionally, whereas *sopD2*, STM4595, and *stjB* were absent. Serovar Paratyphi B dT+ and serovar Duisburg showed the second highest similarity. Nine of the 104 virulence determinants were different.

The two serovar Livingstone strains shared with serovar 4,12:d:− the same set of fimbrial genes, including an additional *lpfD*. Serovar Duisburg and serovar Paratyphi B dT+ lacked fimbrial set *stjB* in comparison to serovar 4,12:d:−. The serovars Stanley and Schwarzengrund harbored other sets of fimbrial markers. Serovar Stanley carried in addition the genes *lpfD*, *stcC*, and *tcfA*, and serovar Schwarzengrund lacked the markers *steB*, *stfE*, and *stjB* but carried *tcfA*. The two serovar Derby strains and the two serovar Infantis strains lacked the markers *stjB* and STM4595. Both serovars also carried *lpfD* and serovar Infantis furthermore *tcfA*. Serovars Enteritidis and Typhimurium encoded more fimbrial clusters than serovar 4,12:d:−. The two serovar Enteritidis strains carried additionally the *lpfD*, *pefA*, Prot6E, *sefA*, and *sefR* fimbrial genes but lacked *stjB*. All five serovar Typhimurium strains carried in

FIG. 2. Virulence determinants microarray data of the 41 strains analyzed. At the top, the strain numbers and serovars, namely, SSW (serovar Schwarzengrund), SST (serovar Stanley), SDU (serovar Duisburg), SEN (serovar Enteritidis), STM (serovar Typhimurium), SIN (serovar Infantis), SLI (serovar Livingstone), SDE (serovar Derby), and SPB dT+ (serovar Paratyphi B dT+), are indicated. In addition, the PFGE type and the VAT of the serovar 4,12:d:− strains are shown. On the left side, the analyzed genes are grouped according to their particular genomic location (SPI-1 to SPI-7; prophages Gifsy1, Gifsy2, Gifsy3, and Fels1; plasmids; islets) or function (fimbrial). The hybridization result of a particular strain is shown by one column within the figure. A white box indicates the absence and a gray box indicates the presence of the target sequence in the strain. BfR, Bundesinstitut für Risikobewertung.

addition *lpfD* and *stcC* and lacked *steB*. Moreover, one serovar Typhimurium strain carried *pefA*.

The recently described *Salmonella* genomic island 1 in various serovars (4) could be only found in serovar Typhimurium phage type DT104 strains. However, *trhH* and the *rep* gene located in *Salmonella* genomic island 1 could be also found in other serovars/strains. The *trhH* gene was also present in serovar 4,12:d:− and serovar Paratyphi B dT+, whereas the *rep* gene in *Salmonella* genomic island 1 could be found in all serovars analyzed.

Other characteristics of serovar 4,12:d:−. All 21 serovar 4,12:d:− strains were negative for genes conferring antimicrobial resistance. Merely two strains were positive for the IS common region 1 (*ISCR1*) element and three strains for the *ISCR3* element. These ISCR elements are associated with the presence of antibiotic resistance (31).

In the microarray, 17 incompatibility group (Inc) marker probes according to Caratolli et al. (6) were present. Inc markers detect the presence of plasmids in strains. The most prevalent Inc group was Inc W, present in 7 of the 21 serovar 4,12:d:− strains. Moreover, one strain showed the presence of Inc FIA, and another strain showed Inc P. Twelve strains showed no Inc markers, indicating the absence of any plasmids.

Three transposases of IS elements were always present, namely SPA2465, IS*200*, and the IS*1351*-like transposase-encoding gene. A fourth transposase, STY343, was detected in 19 of the 21 isolates. Other transposase-encoding genes were found only sporadically or were completely absent (see Table S4 in the supplemental material).

In comparison to serovar Typhimurium, serovar 4,12:d:− strains lacked several metabolism genes, namely, the genes *dgoA*, *hsdM*, *oafA*, STM1896, and STM4497. STM3782 was only in 15 out of 21 strains present. The *pflD* gene was present in all serovar 4,12:d:− strains isolated from humans, whereas it was absent in all serovar 4,12:d:− strains isolated from other origins.

DISCUSSION

PFGE and microarray data presented in this study showed that serovar 4,12:d:− possesses a highly clonal structure. The serovar spreads successfully in poultry and can sporadically cause salmonellosis in humans. Serovar Paratyphi B dT+, which was the second most prevalent serovar in German broilers in 2005 to 2006 (8), has previously been described as a clonal serovar as well (21). However, in contrast to the multidrug-resistant serovar Paratyphi B dT+ clone, the 56 serovar 4,12:d:− isolates investigated in this study were shown to be completely susceptible to antimicrobial agents by phenotypic and genotypic methods. This is difficult to understand, because resistance determinants can easily spread by horizontal gene transfer (29). Consequently, cohabitating serovars from poultry which are under similar selective pressure caused by the use of antibiotics for poultry production could disseminate resistance determinants leading to similar, if not identical, phenotypes. This observation has to be elucidated in the future. Possibly, either a genetic barrier hampers the acquisition of resistance determinants into the serovar 4,12:d:− genome, or the flocks infected with this serovar are not under selective pressure because of the prudent-use guidelines for the minimal use of antimicrobial substances. Unfortunately, no correlation between the prevalences of multidrug-resistant serovar Paratyphi B dT+ and serovar 4,12:d:− with respect to antimicrobial usage can be given.

Data also indicate that serovar 4,12:d:− evolved as a discrete serovar and has not solely emerged by the deletion of the phase 2 flagellar antigen-encoding region of a diphasic serovar. Various genetic differences between serovar 4,12:d:− and serovars which had the same somatic (O) antigen and phase 1 flagellar (H1) antigen as serovar 4,12:d:− but express in addition a phase 2 (H2) flagellar antigen could be observed, especially virulence markers encoded by prophages (Fig. 2). A discrete serovar formation is also supported by the finding that the SPI-3 3′ region of serovar 4,12:d:− is truncated compared to those of the other serovars. In all serovar 4,12:d:− isolates analyzed, the genes *sugR* and *rhuM* were absent. Amavist et al. (1) have described several variations in SPI-3 in different serovars. They concluded that the acquisition of the *sugR-rhuM* region is likely a relatively recent event. The virulence gene content of serovar 4,12:d:− was most similar to that of serovar Derby (seven virulence determinants different), although it is rarely isolated from poultry. No similarity between serovar Derby and serovar 4,12:d:− could be recognized in the PFGE profile (Fig. 1). Consequently, these data do not support any close relationship to serovar Derby. In further studies, the number of strains and serovars for comparison of their gene repertoire to the serovar 4,12:d:− repertoire will be expanded to confirm these initial results.

The virulence gene repertoire of serovar 4,12:d:− showed that all five *Salmonella* pathogenicity islands were present. The most striking result was the complete absence of any virulence determinants encoded by prophages and the absence of plasmids in the majority of the strains. It has been proven that a virulence plasmid bearing the operon *spv* can be necessary to cause severe systemic disease (15). In addition, the *lpf* fimbrial operon encoding the long polar fimbriae was absent in all strains. It was shown that the long polar fimbriae of serovar Typhimurium mediate adhesion to murine Peyer's patches and are required for full virulence (3). Fimbriae are responsible for the initial adhesion of the bacterium to the eukaryotic cells. They are frequently highly host specific and therefore an obvious factor that potentially influences host range. The absence of fimbrial clusters in serovar 4,12:d:− might be a reason for the successful spread of the serovar especially in poultry and the low prevalence in pigs, cattle, and humans. However, fimbrial clusters which showed effects on intestinal colonization and persistence in mice were present in serovar 4,12:d:− (34).

The impact of feed contaminated with serovar 4,12:d:− on the infection of poultry seems to be low but cannot be excluded. The percentage of serovar 4,12:d:− strains isolated from feed received at the NRL-Salmonella was only 1.4% during the last 10 years. However, identical PFGE and VAT profiles were found in isolates from feed and broilers, indicating the possible spread of this serovar by feeding stuffs, but it remains unclear if they are contaminated at the feed mill or secondarily at the poultry premise.

Microarray data revealed that serovar 4,12:d:− strains lacked several metabolism genes. Especially, *dgoA* was absent in all serovar 4,12:d:− strains, and *pflD* was absent in all serovar 4,12:d:− strains isolated from broilers, pigs, and feed but

was present in serovar 4,12:d:− strains isolated from humans. The *dgoA* gene encodes 2-oxo-3-deoxygalactonate-6-phosphate aldolase-galactonate dehydratase forming D-glyceraldehyde-3P involved in galactonate metabolism. The role of *dgoA* is currently not well understood. The lack of this enzyme could have a toxic effect as shown for *E. coli* K12 (16) because of 2-keto-3-deoxy-D-galactonate 6-phosphate accumulation. Moreover, a lack of this enzyme could have a negative effect on the energy production of strains, leading to a slower cell growth because it hampers the production of D-glyceraldehyde-3P, an important intermediate product of glycolysis and glyconeogenesis (27). A slower growth of these strains could also be caused by the absence of the pyruvate formate lyase encoded by *pflD* in serovar 4,12:d:−. This enzyme catalyzes the formation of acetyl-coenzyme A in the presence of pyruvate (28). Another metabolic gene, STM3782, was absent in 6 out of 21 strains. This gene is involved in the phosphotransferase system. A mutation analysis showed a significantly lower intracellular growth rate for serovar Typhimurium compromised by a phosphotransferase system mutation in mice (14).

Any genes which were preferentially present in serovar 4,12:d:− isolates from animals could not be identified.

In conclusion, serovar 4,12:d:− lacked several genes with known contributions to pathogenicity, metabolism, and antimicrobial resistance in comparison to serovars which are highly prevalent in humans and animals, e.g., serovar Enteritidis or serovar Typhimurium. The absence of such genes might cause the low infection rate in humans, although the prevalence in German broilers is high. Apparently, serovar 4,12:d:− possesses genetic factors which facilitate the colonization of broilers. This hypothesis is supported by the observation that the most prevalent PFGE profile 1 in broilers was only rarely found in human isolates. However, identical serovar 4,12:d:− virulence array types were observed in isolates from both hosts. The analysis of serovar 4,12:d:− strains isolated 10 years ago and contemporary isolates showed a low genetic diversity being a sign of the persistence of a highly clonal line in German broilers. Altogether, epidemiological and molecular data show that serovar 4,12:d:− can pass through the food chain from feed to poultry and finally to humans occasionally causing salmonellosis. The virulence and resistance gene repertoire of serovar 4,12:d:− currently does not give reasons to expect that the serovar will pose a similar risk to consumers like other poultry-associated serovars, especially serovar Enteritidis, serovar Infantis, or serovar Hadar. However, serovar 4,12:d:− should be under supervision by public health and veterinary institutes. This will ensure the detection of the spread of zoonosis into other countries and possibly identify changes to a higher prevalence in humans.

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